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CONCENTRATION AND PURIFICATION OF VIRUSES WITH SPECIAL REFERENCE TO REOVIRUSES[1]

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INTRODUCTION

Among the powerful tools employed by virologists and their colleagues, molecular biologists, are those acquired from physical and biological chemists, which allow isolation of macromolecules in their active forms. These techniques, namely, ultracentrifugation, adsorption chromatography, electrophoresis, and partition in liquid phases, have separated complete virus particles (virions) from incomplete fragments (protein capsomeres and nucleic acid) and other subfractions of cells. Concentration of viruses has been necessary to obtain the multiplicity of virus to cell needed for careful study of virus synthesis within cells. It has permitted extrapolation of events measured in a culture of 10^8 cells to events in a single cell. It has, moreover, been vital to biochemical study, and will probably become increasingly important in production of vaccines free from materials with possible harmful effects.

These techniques and a selected few of their applications will be reviewed. Some of my work with reoviruses will be reported.

METHODS

Ultracentrifugation

Differential centrifugation and ultracentrifugation at velocities of 600 to 100,000 $\times$ g are used to pellet materials as large as nuclei and membranes and as small as Picornaviruses. [Picornaviruses are small ribonucleic acid (RNA) viruses with cubic symmetry (33), composed of protein subunits and a single nucleic acid molecule. Enteroviruses and rhinoviruses comprise this group, and have an average diameter of 150 to 300 A.] Angle rotors are usually used to pellet particles. A combination of procedures, several of which will be described, is usually employed to attain final concentration and purity.

Density-gradient centrifugation has become one of the most popular of these methods (21). Swinging-bucket rotors are usually used to minimize "wall effects" (2). In solutions of proper ionic strength and pH which maintain the integrity of the virus, sucrose gradients separate molecules largely on the basis of size, density, and molecular weight. The ideal material for preparing density-gradient columns should be chemically inert, readily available, and of high molecular weight, and should give solutions of high density and low viscosity. No such ideal has been found, but sucrose, for example, in 5 to 20% concentrations, has been used to advantage. Glycerine (with an even higher viscosity than sucrose) is convenient when electron microscopy is planned because of its volatility. Other solutions that have been employed are deuterium oxide with sucrose, diodon, Ficoll, polyvinyl pyrrolidone, or ethanol in water.

It is essential that virus particles be dispersed to be separated from other materials in rate zonal centrifugation (7). Dispersion of aggre-

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gated virus may be effected by a number of surface-active agents. For instance, the anionic synthetic detergent Igepon T-73, 0.1% (sodium *N*-methyl *N* oleoyl taurate), dispersed 90% of previcusly aggregated barley stripe mosaic virus (6).

Density gradients may be prepared in suitable devices, or by layering solutions of several densities upon each other, and allowing diffusion over several hours to form a smooth gradient. Gradient columns have an upper limit to the amounts of materials that can be adequately separated, and generally the smaller the volume in which the particles to be separated are floated onto the preformed gradient, the better. After addition of the crude virus suspension onto the density gradient, centrifugation should be begun as soon as possible to avoid droplet sedimentation ("streaming effects"). Other centrifugation artifacts in addition to "streaming" and "wall effects" may involve "turnover" with layered solutions, "swirling" especially at acceleration and deceleration, "convection," and "aerodynamic effects." "Convection" may be minimized by working at 0 to 4 F, and "aerodynamic effects" may be decreased by keeping centrifuge tubes closed at the top (2). Using sucrose or glycerol density gradients, Williams, Kass, and Knight (34) separated Shope papilloma virus into a highly infectious band and a less infectious lighter zone. With phosphotungstic acid negative staining of these preparations, electron micrographs showed that the lighter band had many coreless forms. This zone was also relatively free from phosphorus on chemical analysis, which was not the case for the infectious band containing nucleic acid. The sedimentation coefficient of particles may be determined in Svedberg units (cm $\times$ 10^{-13} per sec per dyne per g) after density-gradient centrifugation. Thus, the sedimentation coefficient of hemocyanin was found to be 104*S*, while the recorded value with the analytical ultracentrifuge was 105*S*. Strain MEF, type 2 poliovirus had a sedimentation coefficient of 165*S*, while turnip yellow mosaic virus had a sedimentation constant of 106*S* (22).

In equilibrium centrifugation, which separates particles on the basis of their densities, a solution of suitable density is prepared, and the virus preparation is either mixed or layered onto the solution (13). Cesium chloride, rubidium chloride, or cesium sulfate solutions are most frequently used, but sodium and potassium bromide have been used occasionally. Densities may be determined in the preparative ultracentrifuge by use of this method. The results compare favorably with those determined in the analytical ultracentrifuge. Proteins (average density 1.27 to 1.43) may be separated from nucleic acid [average densities: deoxyribonucleic acid (DNA), 1.71; RNA, 2.0] Densities depend somewhat on the experimental conditions of the study. One of the components (sucrose, water, or salt) may be preferentially absorbed by the virus ("solvation effect"), which accounts for slightly altered densities.

Many animal viruses have densities of about 1.30. Two strains of herpes simplex virus with different plaque morphology have been separated on the basis of their differing buoyant densities in cesium chloride. The macroplaque virus had a density of 1.260, and the microplaque clone had a density of 1.27 (25).

With either gradient or equilibrium ultracentrifugation, degradation of virus may occur as it is purified during the procedure. Sucrose, proteins, amino acids, and divalent cations have been found to be protective agents. In a study of potato yellow-dwarf virus, 0.1 M glycine and 0.01 M magnesium chloride were found to be suitable stablizers (4).

Chromatography

The techniques of adsorption and adsorption chromatography usually employ ion-exchange resins or salts prepared in columns for ready collection of the purified eluates. Cellulose ion exchangers are also frequently used. Common cellulose anion exchangers are DEAE (diethylaminoethyl) and ECTEOLA (contains basic groups derived from triethanolamine). The most frequently used cellulose cationic exchanger is carboxymethyl cellulose. Amberlite IRC-50 and Dowex-50 are other cationic exchangers, and Dowex-2 is another anionic exchanger. Chromatographic columns can be used repeatedly, and suffer no apparent deterioration upon months of storage in a cold room in contact with aqueous solutions ranging from 0.05 M sodium monophosphate to 1 N sodium hydroxide. Prolonged exposure to solutions more acid than pH 4 is, however, to be avoided (23).

By use of DEAE cellulose, 90% of the protein

from vesicular fluids from foot pads, infected with foot-and-mouth disease virus, was removed, with near quantitative recovery of both infectivity and complement-fixing activity (8). DEAE chromatography easily separated infectious vaccinia virus from the hemagglutinating antigen produced on infected chorioallantoic membranes (18). Successful applications with many viruses are available, such as influenza (20) and Theiler's virus (17) with amberlite, and poliovirus with Dowex (30).

Woods and Robbins (35) used adsorption and elution from an aluminum hydroxide gel to separate virulent type 1 polioviruses from attenuated strains of the same type. They postulated that these properties reflected differences in structure of the protein portion of the virus particle, a possible genetic attribute.

Youngner and Noll (36) concentrated viruses and studied virus-lipid interactions with cholesterol columns. Influenza virus, Newcastle disease virus, vaccinia virus, and coliphages adsorped to these columns, while many protein impurities did not. Poliovirus also did not adsorb.

Levintow and Darnell (16) utilized rotary evaporation to decrease fluid volume, followed by sequential column chromatography with ECTEOLA and equilibrium-density ultracentrifugation in cesium chloride to produce highly purified poliovirus type 1, in 1- to 5-mg quantities from the S_3 clone of HeLa cells growing in suspension cultures. The growth of cells in suspension greatly increased the numbers of cells which could be easily handled. Their purified virus had a titer of 5×10^{10} plaque-forming units (PFU) per ml.

Electrophoresis

Zone electrophoresis is an exact method for isolating viruses and their components, but it has not been widely used in this manner because of the limit in the amounts of materials that may be so analyzed (3). However, early effective uses of electrophoresis were obtained in purifying two plant viruses, wheat streak mosaic virus (5) and cucumber mosaic virus (31). After isolation (19, 26) and hydrolysis of the nucleic acids of viruses, their base compositions can be accurately determined by electrophoresis (27).

Partition of Liquid Phases

Phase separations with organic solvents offer experimental simplicity and serve as a useful complement to centrifugation methods. Surface properties and differential solubilities are the main determining factors. Phase systems should be as inert as possible to the particles which are to be fractionated. Consideration must be paid to water content, ionic strength, osmotic pressure, denaturing effects on proteins, and ability to dissolve out substances. A low interfacial tension is desirable. Dextron, polyethylene glycols, polyvinyl pyrrilidon, and various alcohols and celluloses have been used (1). Fluorocarbon (Genetron 113) also is used to remove many proteins from crude virus preparations.

A 20- to 100-fold increase of the specific virus activity per milligram of protein of ECHO 7 has been achieved with the organic solvents 2-ethoxyethanol and 2-butoxyethanol by liquid phase partition. Concentrated material had a final titer of 3×10^8 PFU per ml. This method is probably generally applicable to enteroviruses, and has been applied to Coxsackie B5 (14).

APPLICATIONS TO REOVIRUSES

In our laboratory, we have been interested in reoviruses, their ecology and biology, and have developed a somewhat different and simple method of producing a highly concentrated purified preparation of reovirus type 2. This has been done previously by other more complicated procedures. Halonen (12) used a combination of ultracentrifugation, fluorocarbon extraction, and cesium chloride equilibrium-density centrifugation to produce relatively pure virus, but the titers were not particularly high. Gomatos and Tamm (11) produced large amounts of highly purified and high-titered reovirus by reducing cell culture volumes (7 liters to 250 ml) by ultrafiltration under reduced pressure followed by ultracentrifugation (78,000 $\times$ *g* for 3 hr) and treatment with the enzymes ribonuclease, deoxyribonuclease, and chymotrypsin. These and other remaining proteins were removed with fluorocarbon, and ultimate purity was obtained by equilibrium centrifugation in cesium chloride and extensive dialysis.

Reoviruses multiply well in L cells growing in monolayer bottles or suspension culture (10). The virus has a long intracellular phase (36 to 72 hr) before newly manufactured virus is re-

TABLE 1. *Incubations of sedimentable reovirus with several reagents**

Sample†	Temp of incubation (2 hr)	Reciprocal of hemagglutination titer	
		Pellet	Supernatant fluid
	C		
Control	4	6	<3
Control	23	12	<3
Deoxyribonuclease	4	6	<3
Ribonuclease	4	12	<3
Trypsin	23	12	<3
Deoxycholate	4	<3	96

* Sediment containing reovirus was incubated in 1 ml of the various solutions for 2 hr with frequent agitation with a Vortex Junior mixer. At the end of this time, debris was again pelleted at 600 × *g* for 10 min (4 C), and the supernatant fluid was removed. The pellet was washed twice with 10 ml of BSS, and was resuspended in 1 ml of BSS. Hemagglutination titers were then done on these final supernatant and pellet preparations.

† Deoxyribonuclease: 100 μg/ml in 0.15 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane, 0.005 M Mg^{++}, pH 7.3; ribonuclease: 100 μg/ml in Earles' balanced salt solution (BSS); trypsin: 0.5% in BSS; deoxycholate: 100 μg/ml in BSS.

leased by rupture of the membrane of the infected cell (24).

At 72 hr after infection of L-48 cells (29) with a type 2 (988) strain of reovirus (15), approximately 90% of the progeny reovirus is still within cells. In our procedure, infected cells are pelleted at 600 × *g* (4 C) at this time, and the suspending medium is discarded. No further volume concentration is necessary.

After disruption (by three cycles of freezing and thawing in acetone and Dry Ice, or by ultrasonic treatment) of the cells, 72 hr after infection a considerable portion of the virus yield is attached to an easily sedimentable (600 × *g* for 10 min) but as yet unidentified lipid-containing organelle in the cell. The association of much of the newly synthesized virus produced in the cytoplasm with material which sediments with the nucleus was unusual, and very different than in poliovirus, which also replicates in the cytoplasm. Spendlove et al. (28), using fluorescent microscopy, and Dales (9), with sectioned electron micrographs, suggested that reoviruses multiply in the cytoplasm in association with the mitotic apparatus of infected cells, namely, their spindles and centrioles. Electron micrographs prepared in this laboratory by use of the negative-staining technique with phosphotungstic acid and osmotically disrupted cells (32) have not definitely confirmed or denied these findings, since with this technique the mitotic apparatus may not be well visualized. It is not clear in our studies as yet whether the mitotic apparatus of the cell has been sedimented with nuclear and membrane debris.

Virus was, however, released from this sedimented debris into the supernatant fluid by incubation with 100 μg/ml of deoxycholate for 2 hr at 4 C. Virus was not released by appropriate incubations with trypsin, ribonuclease, or deoxyribonuclease, indicating that the sedimented virus was attached to lipid (Table 1).

Debris from which reovirus had been leached was removed by centrifugation at 800 × *g* for 15 min at 4 C. The supernatant fluid then contained the concentrated and partially purified preparation. The results of two representative experiments are shown in Table 2. Hemagglutination titers of 820,000 to 1,640,000 per ml were achieved repeatedly. Further purification of the preparation was accomplished by sucrose density-gradient ultracentrifugation (Spinco model L;

TABLE 2. *Concentration of reovirus-2 from 10 (32-oz) monolayer cultures of L cells*

Determination	Expt 1	Expt 2
Number of L-cells infected	2×10^8	2×10^8
Virus input		
Hemagglutination titer (HA)	3.2×10^5	3.2×10^5
TCD_{50}*	3×10^8	3×10^8
Volume (ml)		
Initial	1,000	1,000
Final	10	10
Virus produced		
Total HA	8.2×10^6	1.64×10^7
Total TCD_{50}	2.4×10^{10}	6×10^{10}
TCD_{50}/cell	120	300

* Expressed as 50% tissue culture infectious doses (TCD_{50}).

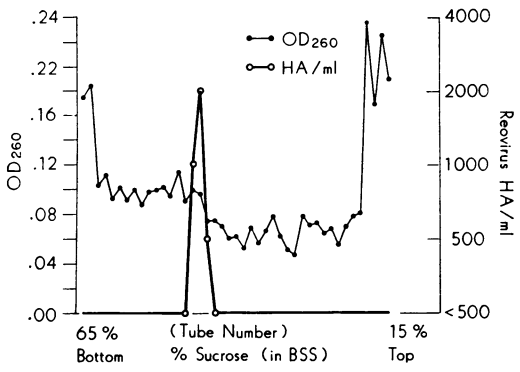

FIG. 1. *A 2-ml amount of the partially purified and concentrated preparation of reovirus-2 was layered onto a 15 to 65% sucrose gradient in BSS. It was centrifuged in a Spinco model L centrifuge at 24,000 rev/min in a SW25 swinging-bucket rotor for 150 min at 0 F. Fractions were collected in 25-drop samples by puncturing the bottom of the tube with a fine needle. Optical density at 260 mμ and hemagglutination were measured.*

Fig. 1). This concentration procedure has proved to be a rapid preparative tool to further study reoviruses.

Ultracentrifugation with or without density gradient analysis, chromatography, electrophoresis, and separation in liquid phases, combined with continued biological insight, has become stock and trade of modern virology.

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